RATIONAL STRUCTURE-BASED DESIGN OF ANTI-BREAST-CANCER DRUGS TARGETING THE ERBB FAMILY OF RECEPTOR TYROSINE KINASES

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The ErbB2 tyrosine kinase domain (TKD) has been implicated in the growth and progression of the most aggressive forms of breast cancer. Breast cancer cells which produce large amounts of ErbB2 result in the excessive activation of multiple signaling cascades within the cell. To date, the crystal structure of ErbB2-TKD domain has yet to be determined. The purpose of this study was to characterize the structure of ErbB2 and identify potential new inhibitors to this domain by modeling, docking and virtual ligand screening (VLS).

Monoclonal antibodies have already demonstrated potent anti-tumor activity against ErbB2, but this approach gives rise to drug-delivery problems. We aimed to simplify the problem of drug-delivery by identifying orally bioavailable organic compounds that can permeate the cell and inhibit the catalytic activity of ErbB2 and hence arrest the progression of the disease. These compounds can also be tailored so they inhibit two or more closely related RTKs.

We have undertaken a unique approach to build a molecular model of the ErbB2-TKD domain using homology modeling with multiple templates, ligand docking and side-chain refinements. The resulting models have been used as ErbB2 structures for VLS.

The inhibitors bound to the ATP binding site in the crystal structure of the modeling templates were used as a docking validation tool. The ligands in each of the templates were 're-docked' into the crystal structure and placed within the ligand binding pocket between 0.2Å and 0.8Å RMSD compared with the crystal structure of each complex. This high accuracy of 're-docking' ligands into TKDs constitutes a validation of our docking methodology and is in perfect agreement with previous results.

A comprehensive library of known ErbB2 and other tyrosine kinase inhibitors was constructed and docked into the ErbB2 models in order to establish a scoring threshold for VLS. A virtual ligand screening of the National Cancer Institute database (more than 127,000 compounds) was performed, and compounds were selected for further analysis. A database enrichment factor of 99% was recorded and is to our knowledge the highest ever-achieved using VLS. These VLS results indicates that our ErbB2 molecular model can provide a foundation for structure-based design of drugs and therefore overcoming the problem of our unsuccessful attempts to solve the crystal structure for this protein.

This work demonstrates that accurate molecular modeling and virtual ligand screening can identify potential 'drug-like' inhibitors that can bind to the receptor and potentially facilitate anti-tumor activity in breast cells.

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FLEXIBLE RECEPTOR MODEL EXPLAINS STRUCTURAL DETERMINANTS FOR AGONIST AND ANTAGONIST ACTIVITY IN RETINOID RECEPTORS

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Until December 2001 we have been working on the rational computer-aided design of targets for Retinoic Receptors (RRs), which has led to the discovery of 2 novel antagonists and 3 novel agonists for the Retinoic Acid Receptor (RAR) (see Ref. 1 below).

The most widely employed therapy against breast cancer is based on Estrogen Receptor (ER) modulators, which have undesirable side effects and are inefficacious against ER-negative breast cancer cells. Compounds targeted to the RRs, members of the Nuclear Receptor (NR) superfamily, are known to inhibit the growth of a wide variety types of cancer, including ER-positive and ER-negative breast cancer cells. RRs are divided into two sub-families, the RAR isotypes (α, β and γ) and the Retinoic X Receptor isotypes (RXR α, β and γ). Both agonists and antagonists of RAR can present anti-tumor activity against breast cancer and also RXR ligands appear to be also efficacious agents against breast cancer cells. Since RXR is also the obligate partner of many other NRs –including itself-, understanding and controlling the structural mechanisms which govern RXR specificity and synergy will also help to design modulators with improved profile against breast cancer cells.

With our collaborators from the Burnham Institute, Dr. Marcia Dawson and Dr. Xiao-Kun Zhang, we have been working upon the elucidation of the structural mechanisms of 15 compounds which confer them agonist or antagonist activity to RARs and RXRα. We have used the experimental crystal structures of the human agonist-bound RARγ and RXRα and the human antagonist-bound RARα. We have also modeled the human antagonist-bound RXRα based on the corresponding murine crystal structure. The ligands were docked to the receptor using a flexible ligand/grid receptor docking algorithm. Further optimization of the receptor was also carried out with energy minimization in which the side chains around the ligand were flexible. The fully flexible docking procedure led to docking scores and calculated binding energies which show close correlation with experimental transcriptional data. The structural analysis of the ligands within the receptors provides a better understanding of the key features which govern agonist or antagonist activity and new avenues toward the optimization of new compounds with improved therapeutical profile against breast cancer.

This study demonstrates that the combination of rational computer-aided methods, chemical synthesis and biological activity tests is a powerful tool for designing new compounds with improved therapeutical profile against breast cancer.

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2. Dawson MI, Cavasotto CN, Jong L, Feng K-C, Leid M, Li H, Abagyan R, Zhang X-K. *93rd AACR Annual Meeting*. San Francisco, California, April 6-10 2002.

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RADIOACTIVE DRUGS BASED ON TARGETING OF SPECIFIC RECEPTORS AND CELL CYCLE STATUS FOR TREATMENT OF BREAST CANCER

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Overexpression of EGFr plays an important role in the progression of breast cancer. The up-regulation of EGFr is positively correlated with poor prognosis. Up to 80% of human breast tumors are androgen receptor-positive. AR levels are negatively correlated with the expression of EGFr. In metastatic tumors ARs are present with twice the frequency of PgRs. One in four tumors expresses AR as a sole sex hormone receptor. 25% of metastatic tumors classified as receptor-negative on the basis of ER and PgR measurements, express ARs. On this basis a series of EGFr- and AR-targeting radiopharmaceuticals toxic only to cells in the S-phase was designed, synthesized and tested in vitro and in vivo. These agents can reach both, EGFr- or AR-expressing cell populations and can evaluate simultaneously in a non-invasive way two parameters, the receptor status and the S-phase fraction.

New agents include constructs of ^{125}IUdR and ^{125}IUdR-5'-phosphates with EGF and dihydrotestosterone incorporating various linkers able to modify the in vivo stability and the rate of ^{125}IUdR release from the carrier. All agents were prepared at a "no-carrier-added" level from their respective trimethylstannyl precursors. In vitro testing included determination of stability, subcellular distribution, binding assays to establish the integrity of receptor-seeking moieties, and metabolite analyses. In vivo experiments determined biodistribution in normal and tumor-bearing mice as well as the subcellular localization of ^{125}I in tumor extirpated at various time points after administration. Selected agents were tested in therapy protocols.

The overall chemical yield of new constructs varies from 90% for DHT constructs to approximately 10% for EGF constructs. The primary reason for lower yields of EGF constructs is the loss of the product during purification procedures. The linkers tested include 5'- and 3'-succinates, derivatives of maleimide, and short peptide linkages for carboxyl modifications. In the absence of cells, all constructs appear to be stable in PBS buffer and cell culture medium for up to 6 hours. The release of ^{125}IUdR is dramatically accelerated in the presence of cells expressing targeted receptors, which indicates intracellular processing of the construct. Stability in the presence of cells depends on the chemical nature of the linker with succinates being the least stable and releasing ^{125}IUdR at the most rapid rate. Maleimide linkers, although less efficient in the preparation of constructs, are the most stable in vitro and in vivo. Clearance of DHT-based agents from systemic circulation is rapid and in therapy studies local administration produces better outcome. Biodistribution of EGF-based drugs indicates transient, high uptake by the liver. The effect of this nonspecific uptake on therapy and potential radiation exposure of normal tissues is under the investigation. Although no cures were observed, the growth of tumors treated with DHT-based ^{125}IUdR is considerably slowed, on average >600% decrease. This effect is competed by the inclusion of high concentrations of DHT in therapy schemes. The tumor growth is still slower than untreated controls but the average decrease amounts to only about 200%.

Cell cycle-dependent radiopharmaceuticals delivered only to tumors expressing specific receptors should provide a novel diagnostic and therapeutic modality for the treatment of breast cancer. Improved medical treatments for breast cancer depend on identification of reliable prognostic indicators and on development of new therapeutic modalities. These studies have a potential to provide both.

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SELECTIVE INHIBITORS OF 17β-HYDROXYSTEROID DEHYDROGENASE

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Hydroxysteroid dehydrogenases (HSD) play an essential role in the biosynthesis and degradation of all steroid hormones. 17β-HSD's are a subclass of HSD isoenzymes that specifically participate in the final steps of the synthesis of estrogens and androgens. Human type 1 17β-HSD, also known as 17β-estradiol dehydrogenase, catalyzes the reduction of estrone to 17β-estradiol, the biologically active estrogen. A compelling case can be made for the development of selective inhibitors of 17β-HSD as an approach to the design of new therapeutics in the treatment of breast cancer. We have observed that the natural product gossypol and its derivatives inhibit 17β-HSD as competitive inhibitors of NAD(P) binding, suggesting that these can be used as lead compounds for structure-based drug design of inhibitors targeted to the Rossman fold. For example, gossylic lactone exhibits a Kd = 4μM. We have recently developed a new class of dehydrogenase inhibitors, dihydroxynaphthoic acids, that are analogs of gossypol. A synthetic scheme has been developed to synthesize a wide range of substituted dihydroxynaphthoic acids and related compounds as potential inhibitors of 17β-HSD.

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ACTIVE SITE ANALYSIS AND RATIONAL DESIGN OF HYDROXYSTEROID DEHYDROGENASE INHIBITORS

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Type 1 17b-Hydroxysteroid dehydrogenase (17b-HSD-1) catalyzes the reduction of the weak human estrogen, estrone, to the more potent 17b-estradiol via the oxidation of NAD(P)H. The role of 17b-estradiol in the stimulation of breast cancer cell growth and the proliferation of certain hormone-sensitive breast cancers makes 17b-HSD-1 an attractive target for drug design. Gossypol, a component of cottonseed oil, is known to be a potent inhibitor of certain dehydrogenases. In the case of 17B-HSD-1, the gossypol derivatives gossylic lactone and gossylic iminolactone exhibit strong competition for cofactor binding with inhibition constants as low as 4mM. In an effort to aid the rational design of improved inhibitors based on theoretical docking studies, an algorithm was implemented in C++ to evaluate the active site surrounding a docked inhibitor. The binding modes of gossylic lactone and gossylic iminolactone were predicted by means of docking to a crystal structure of 17B-HSD-1. These results were used to analyze the active site around the inhibitors, generating new theoretical inhibitors. Promising inhibitors were selected based on synthetic schemes and assessment of predicted potency and selectivity.

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SYNTHESIS AND CHARACTERIZATION OF THE FIRST-GENERATION CYTOTOXIC ANTIESTROGENS AND THEIR CULTURE EVALUATION

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Research Goal: The purpose of this presentation is to examine new agents that combine a transition metal anticancer agent (CDDP and its analog) portion with an antiestrogen (tamoxifen and its analogs) portion in cell cultures of breast and ovarian cancer cell lines.

Hypothesis: The tamoxifen portion of the conjugate serves as a carrier to selectively accumulate CDDP portion in the nucleus of estrogen receptor (ER)-positive cancer cells, while CDDP alone does not show any selectivity between normal and cancer cells. The combined agent would result in selective accumulation in ER-positive cancer cells and also produce a cytotoxic effect by the reaction of CDDP with DNA.

Background: It has been established that therapeutic effect of the antiestrogen agent tamoxifen is through its interaction with estrogen receptors (ER), which is located at the cell nucleus. The cytotoxic effect of CDDP comes from its interaction with DNA. No selectivity was observed between normal and cancer cells. Synergistic inhibition of human cancer cell growth by CDDP and tamoxifen on an intermolecular basis has been demonstrated in vivo.

Methods: A number of conjugates between CDDP and tamoxifen have been designed, synthesized, and characterized. The anti-tumor activity of these conjugates were evaluated in cell culture studies of breast and ovarian cancer cell lines using MTT methods.

Results and Discussion: All conjugates inhibit the growth of breast and ovarian cancer cell lines. The IC50 values of the conjugates are larger than that of tamoxifen, but comparable with that of carboplatin on breast cancer cell line MCF-7. However, the potency of the conjugates varies a lot on ovarian cancer cell lines. For selected cell lines, the IC50 values of the conjugates are larger than that of tamoxifen, but smaller than that of carboplatin. A platinum (IV) complex tethered with hydroxy group showed higher potency than CDDP on MCF-7 cell line.

Conclusion: The conjugates show potency between tamoxifen and carboplatin. No synergistic effect was observed for the first generation conjugates. However, no binding affinity of the conjugates with ER receptors has been measured, and maintenance of the integrity of the conjugates was not established in the cell culture studies.

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MECHANISMS OF ACTION OF TAXOL-LIKE DRUGS AT LOW CONCENTRATIONS

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Previous studies with lung carcinoma A549 cells found that low concentrations of Taxol inhibited cell proliferation and induced a large population of hypodiploid cells without blocking cells at mitosis. We report here that low concentrations of microtubule-stabilizing agents epothilone B and discodermolide also produce aneuploid populations of cells in the absence of a mitotic block. Such aneuploid populations are diminished in an epothilone B-resistant cell line. In contrast, microtubule-destabilizing agents like colchicine, nocodazole and vinblastine are unable to initiate aneuploidy. The aneuploid cells result from aberrant mitosis, since multipolar spindles are induced by the stabilizing drugs, but not by destabilizing agents. Induction of aneuploid cells by low concentrations of Taxol and epothilone B is accompanied by the increased expression of p53 and p21, supporting that the cells from aberrant mitosis are arrested at G1 phase. Gene expression in A549 cells treated with epothilone B (2.4, 5, and 10 nM) and Taxol (4, 8 and 45 nM) is explored using Affymetrix microarray chips. A group of stress response genes are significantly up-regulated by low concentrations of Taxol and epothilone B. The effect is less significant at a higher concnetration of Taxol (45 nM) that results in a mitotic block. The results suggest that the mechanism underlying aberrant mitosis may not be the same as that responsible for mitotic block. We hypothesize that cells treated with low concentrations of Taxol-like drugs may inherit DNA damages after aberrant mitosis, and this determines the sensitivity of cells to Taxol-like drugs. This study will help us to better understand the mechanisms of action of Taxol, an approved drug for the treatment of breast cancer.

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THERAPEUTIC ACTIONS OF NOVEL POLYAMINE ANALOGUES AGAINST BREAST CANCER

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The intracellular polyamines, spermidine, spermine, and putrescine, play an important role in the proliferation and death of normal and malignant cells. As a consequence work has focused on development of inhibitors of this metabolic pathway. The initial purpose of this work was to evaluate the action of one polyamine analogue, DENSpm (N1-N11-diethylnorspermine in breast cancer through preclinical models and a phase II trial in women with stage IV breast cancer.

An open-label single-center phase II study of DENSpm in women with stage IV breast cancer was conducted between April 2000 and December 2001. Patients received DENSpm at 100 mg/m2 IV days 1-5 repeated every 21 days. The primary study objective was to estimate if at least 20% of patients were progression-free at 4 months. A two-stage design was planned. A total of 16 patients were enrolled on the first phase. There was no significant toxicity except for two patients who required brief hospitalization for grade 3 abdominal pain. The sixteen evaluable patients received a total of 43 cycles but no patient had stable disease at 4 months. Thus the study did not proceed to the second stage of accrual. (Wolff AC et al, Breast Cancer Res Treat 69:286, 2001).

As a consequence recent preclinical studies supported through this grant have focused largely on other polyamine analogues that might move into clinical studies. The activity of two analogues (CPENSpm and CHENSpm) to synergize with conventional cytotoxics was evaluated in human breast cancer cell lines. Two cytotoxics, vinorelbine and the fluoropyrimidines, showed the most promise, and CPENSpm was the more active agent in these combinations. (Hahm HA et al, Clinical Cancer Res 7:391-9, 2001). In addition, new conformationally constrained polyamine analogues and oligoamines were shown to inhibit growth and induce apoptosis in human breast cancer cell lines, and several inhibited the growth of MDA-MB-231 cell xenografts in nude mice (Huang Y et al Proc Amer Assoc Cancer Res 43:90, 2002). These encouraging preclinical data justify further evaluation of polyamine analogues for treatment and prevention of breast cancer.

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SOLUTION-PHASE AND SOLID-PHASE SYNTHETIC APPROACHES TOWARD THE PREPARATION OF LAULIMALIDE ANALOGS

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The paclitaxel-like microtubule stabilizing agents the epothilones, discodermolide, and the eleutherobins have generated great excitement as potential agents for the treatment of various cancers, including breast cancer. While generally thought to act via a mechanism similar to that of paclitaxel, these "second generation" microtubule-stabilizing agents offer potential advantages, such as improved solubility and efficacy toward drug-resistant tumors. The marine macrolide laulimalide was recently identified as a new member of this exclusive group of antimitotic agents.

Our project has targeted the development of efficient strategies for the preparation of laulimalide and structural analogs of laulimalide for biological evaluation. To this end, we have developed a solution phase synthesis that provides the natural product, but is highly amenable to integration of structural variations into the molecule. We have specifically focused on the preparation of analogs that will accomplish three goals: 1) improvement of the chemical stability of the biologically active compound; 2) structural simplification leading to increased ease of preparation; and 3) the development of a working understanding of the structural constraints for biological activity.

To allow the efficient preparation of a library of laulimalide analogs, we are developing solid-phase combinatorial methods for the assembly of modified substructures. Our solution-phase syntheses of laulimalide and laulimalide analog substructures, along with our progress toward the implementation of our combinatorial synthetic approach will be presented.

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A STRUCTURE-BASED SOLID-PHASE SYNTHESIS APPROACH TO THE DEVELOPMENT OF NOVEL SELECTIVE ESTROGEN RECEPTOR MODULATING STEROIDS

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Objective: The project undertook the development of new steroidal chemotherapeutic agents utilizing a solid-phase synthesis approach.

Specific Aims:
1. Preparation of resin bound stannylated steroid intermediate.
2. Synthesis and characterization of mono-substituted estrogens, derivatives of aminomethylphenyl and carboxamidophenyl estrogens.
3. Evaluate new derivatives as estrogen receptor binding agents.
4. Prepare second generation estradiol derivatives based on biological results.

Results:
The results of this project are summarized as follows. We were able to prepare the stannylated estradiol and link it to a carboxy resin via the 3-hydroxyl group. Initially we demonstrated the feasibility of the Stille coupling approach using mono-substituted aryl iodides. Subsequently we prepared the resin bound 3-aminomethyl phenylvinyl estradiol and converted it to a series of 3-acylaminomethyl phenylvinyl estradiols. Similarly we prepared the 4-carboxy phenylvinyl estradiol and converted it to a series of 4-carboxamido phenylvinyl esradiols. These series were evaluated for their affinity for the estrogen receptor (alpha)-ligand binding domain (ERα-LBD). Relative binding affinities (RBA) were generally significantly lower than estradiol and the underivatized compounds but still demonstrated estrogenic effects. Based upon these results we have undertaken the synthesis of analogs utilizing a modification (convergent synthesis) of the initial approach.

The biological data were correlated with the structures using NMR conformational analysis and by molecular modeling. The NMR studies indicated that the derivatives existed in solution in an equilibrium between two low energy conformers. These conformers were among those identified by molecular modeling and were docked with the crystal structure for the ER-LBD. The model suggested that the 17α-substituents were tolerated by the receptor and were in a position to affect the orientation of the key helix-12 of the protein. These finding supported our further synthetic efforts toward preparing estrogen receptor antagonists and modulators.

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SOLID-PHASE COMBINATORIAL APPROACH TO ESTRADIOL TAMOXIFEN/RALOXIFEN HYBRIDS: NOVEL CHEMOTHERAPEUTIC/PROPHYLACTIC SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERM)

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Objective: The project undertook the identification and preliminary development of new chemotherapeutic agents for the treatment and/or prevention of breast cancer.

Specific Aims:

1. Design based on the docking of target compounds with crystal structure of estrogen receptor-ligand binding domain (ER-LBD).
2. Synthesis of new 17α-E/Z-[(dialkylaminoalkoxy)phenyl vinyl estradiols and their characterization.
3. Biological evaluation as ligands for the ER-LBD as well as cell proliferation and uterotrophic growth assays.
4. Analysis of biological results to design second generation agents.

Results: During the past 18 months we have worked on Specific Aims #1-3. As part of an ongoing program we have developed models to describe the interactions of our 17□-(substituted-phenyl)vinyl estradiols with the ER-LBD. Our initial studies have focused on simple mono-substituted derivatives because of the conformational mobility around the phenyl-vinyl axis. These studies indicated that there probably existed substantial steric toleance at that binding site that would accommodate large groups, such as the proposed dialkylaminoalkoxy ethers. We had previously worked out some of the technology related to the solid-phase synthesis procedures and concentrated on the specific reactions related to the incorporation of the substituted aryl ethers. We have done most of this chemistry in solution -phase before we extend it to solid -phase synthesis. We also had to prepare and characterize the dialkylaminoalkoxy aryl iodides. We have prepared most of the ortho-, meta-, and para- iodo derivatives in which the alkyl chain conatins 2-4 carbons and the dialkyl amino group is acyclic (diethyl-) or cyclic (pyrrolidino- and piperidino-). This will give us a substantial variation in properties among the target compounds. Our biological assay system has been developed to enable us to evaluate the compounds not only for their ability to bind to the receptor but to stimulate or block it. Preliminary results have been generated with a training set to validate the approach.

Conclusions: The project has completed several of the specific aims and is on schedule to complete the synthesis and evaluation of the preliminary library of target compounds. The availability of the biological assays.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0384 supported this work.

STRUCTURE-ACTIVITY RELATIONSHIPS IN TACCALONOLIDE A, A MICROTUBULE-STABILIZING AGENT

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In the course of a previous research program aimed at the discovery of new microtubule poisons, we had isolated from Tacca chantrieri the unusual steroid taccalonolide A.

This material displayed a taxol-like effect on the microtubule network of mammalian cells as shown by indirect immunofluorescence procedures and in cell-free systems. Although the natural product is considerably less potent than other compounds displaying this mechanism of action, it was interesting because it showed activity against multi-drug resistant cells overexpressing the p150 drug-efflux pump.

Taccalonolide A is a polyoxygenated steroid in which the usual side chain is cyclized onto the five-membered D-ring to form two additional rings. We are examining the structure-activity relationship by functional group deletions and more deep-seated structure modifications. Results of this work helps to define the minimal structural requirements for the microtubule-stabilizing effect of taccalonolide and may lead to a more potent derivative for further drug development.

In a first round of modifications we modified the three electrophilic sites in the molecule by saturation of the ring-E double bond, deoxygenation of the ring-A epoxide and reduction of the ring-B ketone, respectively.

In a second round we are deoxygenating ring A completely as well as deleting rings A and F, respectively.

Development of multi-drug resistance against commonly used chemotherapeutic agents is observed frequently in clinical settings and invariably worsens the prognosis for the patient. Hence the development of agents that are not subject to or can overcome such resistance is a high priority.

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DESIGN AND SYNTHESES OF DUAL-ACTING INHIBITORS FOR BREAST CANCER

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Estrogen levels in breast tumors of post-menopausal women are at least ten times higher than estrogen levels in plasma. The high levels of estrogen in these tumors are presumably due to in situ formation of estrogen, possibly through conversion of estrone sulfate to estrone by the enzyme estrone sulfatase. Therefore, inhibitors of estrone sulfatase are potential agents for the treatment of estrogen-dependent breast cancers. Among all the estrone sulfatase inhibitors, estrone-3-O-sulfamate (EMATE) and its analogs are the most potent. EMATE is classified as an active-site directed irreversible inhibitor. Recently, non-steroidal estrone sulfatase inhibitors were developed based on the fact that EMATE was found to be estrogenic. Non-steroidal sulfatase inhibitors such as coumarin sulfamate and (p-O-sulfamoyl)-N-tetradecanoyl tyramine were reported to inactivate estrone sulfatase in an active-site directed manner. It can be concluded that the common functionality for sulfatase inactivation is a phenylsulfamoyl group.

We synthesized (E)-4-hydroxytamoxifen sulfamate as dual inhibitor (inhibitor with sulfatase inhibitor activity and antiestrogenic activity). (E)-Hydroxytamoxifen sulfamate competitively inhibited estrone sulfatase and exhibited apparent Ki of 35.9 ± 4.4 micromolar. It has higher affinity than the substrate estrone sulfate since the Km of the substrate is 90.2 ± 8.0 micromolar. Eight sulfamate analogs with nafoxidine nucleus were synthesized. In addition, four sulfamate analogs with raloxifene nucleus were also synthesized as dual acting agents.

The dual inhibitors were tested for their abilities to inhibit estrone sulfatase activity of rat liver microsomes. The most potent nafoxidine sulfamate and raloxifene sulfamate are 10 (Ki = 4 micromolar) and 60 times (Ki = 60 nanomolar) respectively more potent than (E)-4-hydroxytamoxifen sulfamate in estrone sulfatase inhibition. The dual inhibitors also inhibit the proliferation of estrogen-dependent breast cancer cell growth stimulated by estrone sulfate.

In conclusion, the newly synthesized inhibitors represent potential agents for the treatment of estrogen-dependent breast cancer.

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DESIGN, SYNTHESIS, AND BIOLOGICAL EVALUATION OF NEW PACLITAXEL PHOTOAFFINITY ANALOGS

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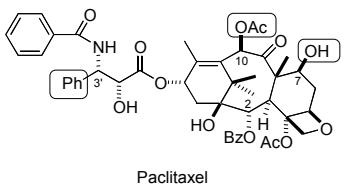
Paclitaxel (taxol®), a structurally complex diterpenoid, has been developed as a potent chemotherapeutic drug against a variety of cancers such as breast cancer, ovarian cancer, and advanced lung cancer. It binds to and stabilizes microtubules disrupting their function. However, the exact paclitaxel binding site at the molecular level is not well characterized, and it is essential to understand the interactions between the molecule and the binding site in order to design and develop second generation analogs of paclitaxel with improved bological profiles.

The photoaffinity labeling approach is a powerful method to identify the paclitaxel binding site and to study paclitaxel-tubulin interactions. By attaching various photoaffinity labels to different positions of the paclitaxel molecule, these photolabeled analogs can be utilized as probes to map the binding site.

Figure 1. **The Structure of Paclitaxel and its Modified Positions**



Paclitaxel

Based on structure-activity relationship (SAR) data, novel photolabeled analogs modified at 3', 7 and the 10 position (Figure **1**) were designed and prepared in good yields starting from paclitaxel itself or its precursor baccatin III using several carefully designed efficient chemistry routes.

Biological evaluation of these new analogs was conducted using our tubulin assembly method. The results demonstrated that these analogs possess excellent activity and are potential candidates for probing the paclitaxel binding site. The results from this research are expected to provide a better understanding of the molecular interactions between paclitaxel and its binding protein. This will eventually facilitate the design of novel paclitaxel analogs.

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TACCALONOLIDE A: A NEW TAXOL-LIKE MICROTUBULE-STABILIZING AGENT

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More effective and less toxic therapies are needed for the treatment of breast cancer. Some of the most effective therapies identified to date for the treatment of metastatic breast cancer include drugs that target cellular microtubules. The goal of this research is to investigate the molecular pharmacology of a new Taxol-like microtubule-stabilizing agent, taccalonolide A. Taccalonolide A is a highly acetylated steroid that has microtubule-stabilizing properties similar to Taxol and it is the first natural steroid to exhibit microtubule-stabilizing activity. Immunofluorescence studies show that cells treated with taccalonolide A exhibit abnormal bundling of interphase microtubules and the formation of abnormal multi-polar mitotic spindles. Taccalonolide A inhibits cellular proliferation of breast cancer cells at low micromolar concentrations and in this regard it differs from the other microtubule stabilizers that are effective at low nanomolar concentrations. Taccalonolide A appears to have an advantage over Taxol in that it is a poorer substrate for transport by P-glycoprotein. As is the case for other agents that disrupt microtubules, taccalonolide A causes Bcl-2 phosphorylation, G2/M arrest, and initiation of apoptosis.

Several studies have shown that the density of tumor blood vessels in the primary tumor serves as a prognostic indicator in breast cancer and suggests that angiogenesis may be critically important in breast cancer. Some microtubule-active drugs have been shown to have antiangiogenic activity, which may explain the differences in therapeutic efficacy of these agents against breast tumors. The ability of drugs to inhibit endothelial cell proliferation and invasion is seen as an indication of their antiangiogenic potential. The antiangiogenic potential of taccalonolide A was assessed in these assays and the data suggest that taccalonolide A may have antiangiogenic activity.

Taccalonolide A represents an exciting new microtubule-stabilizing agent that appears to differ from Taxol with regard to its potency, effectiveness against multidrug resistant breast cancer cells, and antiangiogenic potential.

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CHARGE DENSITY STUDIES OF THREE ESTRADIOL SYSTEMS

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It has been shown that the development of certain types of cancer can be hormone dependent. Specifically, the initiation and progress of the majority of breast cancer cases are influenced by a family of hormones called estrogens. Estrogens, such as estradiol, have the ability to bind as ligands to the estrogen receptor in the first of many steps which could result in the activation (agonistic effect) or repression (antagonistic effect) of genes critical in the mechanism of tumor growth. While the mechanism by which the estrogens influence cancer is currently unknown, subtle changes in the chemical structure of estradiol and the other estrogens are known to elicit different biological responses in the development of cancer.

Biological molecules contain atoms bonded together by electrons in a particular manner to impart distinct chemical reactions and associations. These characteristic bonds represent, for each molecule, a unique electron distribution (charge density distribution). It has been suggested that the agonistic/antagonistic responses of the different estrogens can be related to such physical properties as their charge density distribution.

Single crystal X-ray diffraction techniques are based on the scattering of X-rays by electrons. This makes it the ideal method for accurately measuring the electron density distribution of a molecule. The advent of new X-ray diffractometers using two dimensional area detectors coupled with new cryogenic techniques allows such data to be collected in a relatively short period of time. More importantly, it now allows comparative electron density studies to be conducted in a reasonable amount of time.

This poster will present the results from charge density studies on three estradiol systems: 17-alpha-estradiol with water, 17-beta-estradiol with methanol, and 17-beta-estradiol with urea. Single crystal x-ray data were collected at 100K on Bruker platform diffractometers equipped with CCD area detectors. Multipole and kappa refinements were performed to model the electron density of the estrogens and the solvent molecules. Comprehensive results of the multipole refinement, calculations of electrostatic potentials, as well as the topological analysis will be presented.

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SYNTHESIS OF DOXORUBICIN-FOLATE CONJUGATES AS NOVEL THERAPEUTICS IN DRUG-RESISTANT BREAST CANCER

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Intrinsic or acquired drug resistance of breast cancer cells to antitumor agents can be mediated by an increased level of expression of membrane efflux systems such as P-glycoprotein that actively remove structurally unrelated chemotherapeutic agents from the intracellular compartment and recycle them to the external environment. As a consequence, treatment of drug-resistant breast carcinoma requires administration of higher doses of antineoplastic drugs, which simultaneously increases the incidence of drug-associated side effects. Conjugation of cytotoxic molecules with folic acid is expected to reduce interaction of the chemotherapeutic agent with membrane efflux systems and to facilitate intracellular accumulation of the chemotherapeutic agent in drug-resistant breast cancer cells.

Synthetic strategies were developed to couple doxorubicin (DOX) via the amino sugar moiety to the carboxyl group of the glutamic acid fragment of folic acid. In addition, a doxorubicin-resistant subline of MDA-MB-231 breast cancer cells was established using selective cloning techniques in the presence of 100 nanoM to 100 microM DOX. Differences in the expression level of the folate receptors between parent and doxorubicin-resistant breast cancer cells (MDA-MB-231/DOX) were assessed by comparative PCR.

Selective coupling of DOX to the gamma-carboxyl group of folic acid using a variety of protective strategies was unsuccessful. Alternatively, a 1:3 mixture of alpha- and gamma-coulpled folic acid/DOX conjugates was obtained using direct coupling of DOX to folic acid in the presence of PyBOP. Incubation of this mixture with the newly generated MDA-MB-231/DOX subline that was 100-fold more resistant to this cytotoxic agent than the parent cell line, significantly decreased the concentration required to kill 50% of the cells in vitro (IC50 = 1.6 microM for DOX and 0.6 microM for folic acid/DOX conjugate, respectively). The expression of the folate receptor as assessed by comparative RT-PCR was 2.5-fold increased in MDA-MB-231 cells when compared to parent cells.

Folate conjugates with cytotoxic agents appear to provide a more effective treatment of drug-resistant breast cancer than administration of unconjugated drugs. Prodrugs of folate conjugates may even be more advantagous.

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CONSTRUCTION OF EPOTHILONE PHOTOAFFINITY LABELS VIA TOTAL SYNTHESIS

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The epothilones have gained worldwide recognition for their excellent biological profiles. The epothilones operate *via* microtubule hyperstabilization, the same mode of action as the highly successful anticancer drug Taxol®. The epothilones display cytotoxicity comparable to Taxol® against several cancer cell lines and are also active against multi-drug resistant cell lines where Taxol®'s activity decreases dramatically. These studies were followed up by successful *in vivo* studies with the epothilones. Presently epothilone B and its aza analogue are in phase II clinical trials for various types of cancers. Phase I clinical trials have also recently commenced for epothilone D. Several other promising analogues are also being evaluated as potential agents for cancer chemotherapy.

Despite the promising biological data obtained thus far, very little is known about the epothilone binding site on tubulin. To aid in these studies, the generation of epothilone photoaffinity labels through total synthesis will be completed. A synthesis of epothilones C and A was carried out first. In this approach, the fragments that were used could easily be modified to generate analogues. In the proposed analogues, hydroxymethyl groups will replace the methyl groups at the C4, C8, and C12 positions and photoaffinity labels will be attached. The thiazole moiety of the epothilones will also be replaced with potential aromatic photoaffinity labels.

The following research has been completed for the epothilone analogues. The total synthesis of the C26 hydroxy epothilone D analogue has been completed and various groups have been attached at this position including aromatic azides and benzophenone. These molecules will be used in photolabeling studies that are being carried out in collaboration with Professor Richard Himes in the Department of Molecular Biosciences at the University of Kansas. Preliminary results on the cytotoxicities and tubulin binding for these analogues were very promising and the photoaffinity labeling studies are presently in progress. The C7-C20 fragment for the synthesis of the C8 hydroxymethyl analogue has been completed as well. Other research to generate the epothilones with various aromatic groups in place of the thiazole ring is in progress.

With these epothilones that contain photolabels at various positions on the molecule, a map of the amino acids involved in their binding to tubulin can be generated. This information can then be used to generate compounds with improved selectivity for the epothilone binding site on tubulin and will provide more potent and selective agents for cancer chemotherapy.

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DISRUPTION OF GROWTH FACTOR FUNCTION BY SYNTHETIC SURFACE-BINDING AGENTS

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The primary focus of this project is the first step in the aberrant cell signaling pathways that lead to uncontrolled proliferation and cancer, namely the interaction of growth factors with their receptor tyrosine kinases (RTKs). Overexpression of RTKs as well as high serum levels of the activating growth factor are seen in certain breast and ovarian carcinomas.

The design of growth factor antagonists that can inhibit ligand-induced receptor activation is a potentially novel route to new anti-cancer drugs. In recent years this strategy has been supported by the development of antibodies (e.g., Herceptin) against RTKs that have been shown to be active against breast cancer in the clinic. The main goals of this project are to design, synthesize and evaluate a novel series of synthetic agents that bind to the surface of growth factors and block their interaction with their RTKs.

In this presentation we report the successful preparation of two classes of protein binding agents. The first involves the attachment of four peptide loops to a central scaffold (based on the calix[4]arene unit). The second is based around a tetraphenylporphyrin unit in which different groups are attached. These structures provide large (~300-500Å2) hydrophobic surfaces for binding to the protein.

In the first series we have prepared a large number of cyclic peptide derivatives based on the 3-aminomethylbenzoic acid group and attached them to the core calixarene scaffold. These have been tested for their ability to interact with platelet derived growth factor using fluorescence spectroscopy, non-denaturing gel electrophoresis and protein binding assays. We have identified one molecule that not only binds to the surface of platelet derived growth factor with high affinity (IC50 < 250nM) under physiological conditions but also blocks its activation of PDGF receptor tyrosine kinase. This molecule also shows potent antitumor activity in a mouse xenograft model of a human cancer that is activated by PDGF.

These compounds have the potential to be the first in a new class of rationally designed antitumor agents that function similarly to new antibody based therapies in blocking growth factor function but that are based around relatively low molecular weight synthetic derivatives.

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1. STUDIES TOWARD SECOND-GENERATION ELEUTHEROBIN SYNTHESIS; 2. SYNTHESIS OF CP-263114 AND CP-225917

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Eleutherobin was first isolated from the soft coral Eleutherobia sp. as a novel marine natural product. It exhibited very potent anti-tumor activities against several breast cancer cell lines. Moreover, eleutherobin has its own advantage that it is more water soluble than Taxol and more effective against some multiple drug resistant or Taxol-resistant cell lines. Reported are studies towards establishing an improved synthesis of eleutherobin.

CP-263,114 and CP-225,917 were discovered from the fermentation broth of a fungus as inhibitors for farnesyl transferase and squalene synthase. Since farnesyl transferase is involved in the functioning of oncogene Ras, these molecules are potential anti-tumor agents. Reported is the conclusion of the total synthesis of these compounds.

Such studies are important since the above compounds have a very limited supply from the natural sources, thus an efficient total synthesis is critical for producing large quantities of materials and their analogs for testing and improvement.

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INHIBITORS OF 17-BETA-HYDROXYSTEROID DEHYDROGENASE TYPE 1 TARGETED TO THE ROSSMANN FOLD

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17-Beta-hydroxysteroid dehydrogenase type 1 (17-beta-HSD-1), also called estradiol dehydrogenase, catalyzes the NADPH-dependent reduction of the weak estrogen, estrone, into the more potent estrogen, estradiol. 17-Beta-HSD-1 is an attractive drug target in hormone sensitive breast cancer. Past efforts to develop selective inhibitors of 17-beta-HSD-1 have focused on design of substrate analogs. It is challenging to develop steroid analogs that are devoid of any undesired biological activity. 17-Beta-HSD-1 is a member of the short chain dehydrogenase/reductase (SDR) superfamily that includes many hydroxysteroid dehydrogenases. Members of the SDR family bind NAD(P)(H) in a motif that is a modified Rossmann fold. We demonstrated previously that the Rossmann folds of classical dehydrogenases can be selectively inhibited by derivatives and analogs of the natural product gossypol. In the present study, we have addressed the question whether the modified Rossmann fold in 17-beta-HSD-1 is a target for identification of lead compounds for structure-based drug design. 17-Beta-HSD-1 was purified from human placenta. 17-Beta-HSD-1 was inhibited by derivatives of gossypol with dissociation constants as low as 4 micromolar. Inhibition was competitive with the binding of NADPH. Molecular modeling studies (AutoDock) using the published coordinates of human 17-beta-HSD-1 suggest that these inhibitors occupy the modified Rossmann fold at the nicotinamide end of the NADPH-binding site, extending towards the substrate site. The results suggest not only that derivatives of gossypol represent attractive lead compounds for structure-based drug design but also suggest that appropriate incorporation of a substrate analog into the design of these Rossmann fold inhibitors may provide Pan-Active Site inhibitors that span the cofactor and substrate site, potentially offering specificity and increased potency.

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IDENTIFICATION OF SYNTHETIC BENZOPYRANONES AS SELECTIVE AGENTS FOR MOLECULAR TARGETS IN BREAST CANCER

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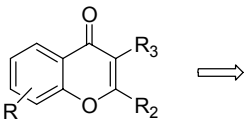
The benzopyranone ring system is the core structure found in a number of natural products such as the flavonoids and isoflavonoids. Substituted 4*H*-1-benzopyran-4-ones have shown activity as protein tyrosine kinase inhibitors, estrogen receptor agonists or antagonists, or inhibitors of steroidogenic enzymes. Initial synthetic chemistry produced a novel synthetic route utilizing readily available salicylic acids and terminal alkynes as starting materials to construct the benzopyranone nucleus. This approach is characterized by mild and high yielding reactions with good functional group tolerance, and is ideal for developing combinatorial libraries centered around the benzopyranone ring system. The hypothesis of this research is that the design, synthesis, and screening of substituted benzopyranone libraries would allow us to utilize the biological potential of these molecules and develop more selective therapeutic agents for molecular targets in breast cancer.



R = Alkyl, OH, OR, NHR, Halogens
R_3, R_2 = aromatic, heteroaromatic, alkyl, cycloalkyl

The 4*H*-1-benzopyran-4-one skeleton is the core structure present in many compounds including estrogenic flavonoids.

The novel solution-phase chemistry developed to synthesize the benzopyranones can be accomplished in several steps. Retrosynthetically, it was envisioned that the benzopyranones could arise from the cyclization of alkynones. Substituted bis-TBDMS-salicylic acids underwent a one-pot acid chlorination-Sonogashira coupling resulting in the synthesis of the critical intermediate, alkynone, in excellent yields. Michael addition of a secondary amine to the alkynone, followed by a 6-*endo-trig* cyclization results in the formation of the six-membered benzopyranone with yields from 70-96%. By using a secondary amine addition to the alkynone, the synthetic strategy prevents the cyclization of the competing five-membered benzofuranone and thus resolves the regioselectivity problem encountered by previous efforts. Synthetic approaches for diversifying the benzopyranone skeleton have also been pursued; substituents at the 3-position on the ring system would dramatically increase the diversity of our library.

The benzopyranone library has been evaluated in initial bioassays (cell proliferation, estrogen receptor binding, and aromatase) using human breast cancer cell lines (MCF-7, MDA-MB-231). This has resulted in agents exhibiting enhanced and differential activities on breast cancer cell growth and aromatase inhibition. Continued synthetic efforts will concentrate on development of more selective agents for molecular targets in breast cancer based upon the benzopyranone nucleus.

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